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Protective effects of HIF-1 in acute, but not chronic, hypoxia: Comparing Madison and Hilltop rats

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1. Summary

In the 1980s a model of adaptive and pathologic responses to acute and chronic hypoxia was described in which Madison (M) rats had pathologic response to acute hypoxia (development of i.e. pulmonary edema) and adaptive responses to chronic hypoxia, where as in contrast, Hilltop (H) rats adapted well to acute but not chronic hypoxia (development of pulmonary edema and hypertension as well as widespread vascular remodelling). The transcription factor Hypoxia-inducible factor-1 (HIF-1) has been extensively investigated and its role as a protective factor has been clearly established. Also, it was indicated that M rats had lower HIF-1 activity compared to H rats under hypoxia.

Thus, we utilized this model to hypothesize that protection from acute hypoxia was associated with elevated HIF-1 activity whereas a prolonged HIF-1 activation would lead to a pathologic response. Cardiopulmonary hemodynamics, blood gasses, right ventricular hypertrophy and pulmonary leak, vascular remodelling, HIF-1 DNA- binding and plasma Epo and VEGF were determined in these animals under acute (24h) or chronic (21d) hypoxic (18,000ft/5500m) or normoxic conditions. Pulmonary HIF-1 DNA-binding activity was unchanged in acute and chronic hypoxia-exposed M rats, but elevated in H rats.

We conclude that pulmonary HIF-1 DNA-binding activity is associated with protection from acute hypoxia, while prolonged activity is associated with pathologic response to chronic hypoxia.

2. Zusammenfassung

In den 1980er Jahren wurde ein Modell von adaptiven und pathologischen Antworten auf akute und chronische Hypoxie beschrieben, in welchem Madison (M) Ratten pathologisch auf akute Hypoxie reagierten (Entwicklung eines Lungenödems) und adaptive Mechanismen bei chronischer Hypoxie zeigten, während Hilltop (H) Ratten sich gut an akute, jedoch nicht an chronische Hypoxie adaptierten (Entwicklung eines Lungenödems, Hypertension, vaskulärer Umbau der Lunge). Hypoxie-induzierter Faktor-1 (HIF-1) wurde bereits intensiv erforscht und seine Rolle als protektiver Faktor klar aufgezeigt. Ebenso zeigten M Ratten unter Hypoxie eine geringere HIF-1 Aktivität als H Ratten.
Deshalb benutzten wir diese Modell um die Hypothese aufzustellen, dass eine gesteigerte pulmonäre HIF-1 Aktivität kurzfristig ein Schutz vor Hypoxie bietet, langfristig aber pathologische Folgen hat. Die beiden Rattenstämme wurden akuter (24 Stunden) oder chronischer (21 Tage) Hypoxie (18,000 Fuss/5500m) oder Normoxie ausgesetzt und anschliessend wurden kardiovaskuläre hämodynamische Messungen durchgeführt und Blutgase gemessen, auf Rechtsherzhypertrophie, Lungenödem und vaskulärer Umbau der Lunge geachtet und HIF-1 DNA-Bindung und Plasma Epo und VEGF gemessen.
Die aufgestellte Hypothese wurde klar von unseren Ergebnissen belegt, HIF-1 schützt vor akuter Hypoxie, eine langfristige gesteigerte Aktivität hat jedoch pathologische Konsequenzen.

3. Introduction

Response to acute or chronic hypoxia can be adaptive or pathologic. Over the past 20 years there has been an explosion of research into the effects of hypoxia on the organism, cells, organelles, and molecules, which has greatly enhanced our understanding of hypoxic responses. However, the majority of research has focused on responses of 'normal', healthy animals or cells, largely because it is not feasible to study humans with pathologic responses to hypoxia at a molecular level.

In the early 1980s, just prior to the recognition of the importance of hypoxia in many diseases, Ou and colleagues identified two rat strains, which presented divergent responses to acute and chronic hypoxia (18, 20). Subsequent studies provided an intriguing story of one rat strain (Madison rats) that developed pulmonary edema in response to acute exposure (24h) to hypoxia (18,000 ft above sea level). Unexpectedly, another strain (Hilltop rats) developed pulmonary edema, severe pulmonary hypertension and pulmonary vascular remodelling in response to chronic exposure to hypoxia (18,000 ft for 21 days) (17-20, 25). In contrast, these Hilltop rats did not develop pulmonary edema in response to acute hypoxia and Madison rats did not develop pulmonary edema in response to chronic hypoxia and had only mild increases in pulmonary artery pressure. Following the discovery of hypoxia-inducible transcription factor-1 (HIF-1), believed to be the long sought oxygen sensor, we (7) decided to re-investigate these models, hypothesizing that HIF-1 activation would be greater in Hilltop compared with Madison rats exposed to acute hypoxia. Indeed, data revealed that there was greater pulmonary HIF-1 activity in the more adapted Hilltop strain than in the edemic Madison strain, leading our old group to speculate that HIF-1 was protective in response to acute hypoxia in this model.

Subsequent research provided evidence that prolonged HIF-1 activity may actually lead to pathologic responses to hypoxia (9, 14, 26). This observation led us to hypothesize that HIF-1 activity may remain elevated during chronic hypoxia in Hilltop rats, but not Madison rats. Chronically elevated HIF-1 activity would be associated with greater pulmonary edema, hypertension and remodelling in Hilltop rats in comparison to Madison rats.

In approaching this research, we discovered the origin of the Hilltop rat strain was in question and it was unclear weather the obtained strain was the same as used in previous studies. Therefore, our initial approach was to verify the validity of the model by exposing Madison and Hilltop rats to acute (24h) and chronic (21d) hypoxia (18,000 ft/5500 m) and evaluating cardiovascular and pulmonary hemodynamics, and blood gases in response to acute and

chronic hypoxia. Hypoxic responses were then evaluated by determining the presence or absence of pulmonary hypertension, edema and vascular remodelling, and right ventricular hypertrophy. Finally, HIF-1 activation was determined by analysing HIF-1 DNA-binding in the lungs and the concentration of HIF-mediated plasma proteins Epo and VEGF.

Keywords: adaptation to high altitude, pulmonary edema, pulmonary vascular remodelling, hypoxia-inducible transcription factor, reduced oxygenation

4. Materials and Methods

Animals

Male Madison Sprague-Dawley (Barrier 217) and Hilltop rats (280–350 g and 10–12 wk of age) were obtained from commercial vendors (Harlan, Indianapolis, IN and Hilltop Rats, Inc., Scottsdale, PA). Animals were allowed *ad libitum* access to food and water, and kept on a 12-hour day–night cycle. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Colorado, Denver, Anschutz Medical Campus.

Experimental Protocol

Animals from each rat strain, Madison (M) and Hilltop (H), were randomly assigned to 24 h or 21 d, normoxic or hypoxic conditions (n=40 total animals, n=5 per group), see Table 1. 18 % oxygen refers to the oxygen content in ambient Denver air.

Chronic Instrumentation

To allow measurement of cardiovascular and pulmonary parameters without the complications of anesthesia, animals in the 24 h protocol were chronically instrumented 48 h prior to implementation of the experimental protocol, as described below. Because catheter patency was unreliable 21 d following instrumentation, cardiovascular and pulmonary parameters were assessed in anesthetized animals under normoxic conditions. Hypothesized changes in response to chronic hypoxia such as pulmonary vascular remodelling and hypertension should be evident despite anesthetic conditions.

Rats were allowed to acclimate to ambient altitude for at least seven days prior to instrumentation. Forty-eight hours prior to surgery, rats were provided water supplemented with acetaminophen/codeine (0.5 mg/ml and 0.05 mg/ml, respectively) for postoperative analgesia. The animals were weighed and hematocrits were determined. Rats were anesthetized with a mixture of ketamine: Rompon (xylazine) (75 mg/kg: 6 mg/kg I.P.). Under aseptic conditions, the left carotid artery was cannulated with a PE-50 (0.58 mm ID; Becton Dickinson) catheter. A PV 1 (0.28 mm ID, Becton Dickinson) catheter with a shallow bend at its tip was inserted into the right ventricle via the right jugular vein and guided into the main pulmonary artery.

Pressure tracings confirmed placement in the pulmonary artery. Next, two PE-50 (0.58 mm ID, Becton Dickinson) catheters were placed in the superior vena cava via the right jugular vein for venous blood collection and to obtain cardiac output values. All catheters were

flushed with heparinized saline, tied off, tunnelled subcutaneously to the dorsal neck region, and exteriorized at the back of the neck. Animals were allowed at least 48 h to recover prior to any treatments. Animals demonstrating signs of infection, diarrhoea, or distress were excluded from study. As discussed above, animals exposed to 21 d hypoxia were anesthetized, catheterized as described above and cardiovascular pulmonary parameters measured under anaesthesia.

Hypoxic Exposure

Animals assigned to the chronic hypoxia groups were exposed to a simulated high altitude: 5,500 m (18,000 ft), barometric pressure - 380 mmHg in a specially designed rodent hypobaric chamber facility as previously described (4, 15).

Hemodynamic Measurements

Animals in the 24 h protocol were placed in a custom designed, small, rectangular, Plexiglas chambers with a portal through which catheters could be passed. Hypoxia was achieved by flushing the chamber with 10% O2 gas (equivalent to 5500 m altitude) and ambient altitude was achieved by allowing room air to circulate in the chamber. Catheters were flushed with heparinized saline and then connected to fluid filled pressure transducers. Systemic blood pressure, pulmonary artery blood pressure, heart rate, cardiac output and blood gases were determined for 10 min under experimental conditions for each animal (normoxia, hypoxia or during anaesthesia in the animals exposed for 21 d). Cardiac output was measured by infusion of Cardiogreen (Sigma Aldrich, St. Louis, MO) dye and a specially designed densitometer and software (Deterministic systems, Boulder, CO) to detect and calculate cardiac output from the dye dilution. Cardiac output was normalized to body weight and reported as cardiac index.

Blood gases and co-oximetry measurements

Blood samples were collected immediately after hemodynamic measurements had been obtained. Arterial blood (0.2 ml) was withdrawn via carotid catheter into blood gas syringes, at which point blood gasses were analysed via ABL5, Radiometer, Copenhagen and co-oximetry via OSM3, Radiometer, Copenhagen, with algorithms specific to rat haemoglobin.

Organ collection and tissue fixation

The left bronchus was cannulated and the left lung fixed with 10% buffered formalin (3 ml) by airway inflation under constant pressure at 25 cm H_2O pressure, after which the heart and lungs were removed en bloc. The hearts were removed and the atria were dissected from ventricles. The right ventricle (RV) and left ventricle plus septum (LV+S) were weighed for assessment of right ventricular hypertrophy (RV/LV+S ratio). Kidneys were placed immediately into 10% formalin. After 18 h the lungs and kidneys were removed from 10% formalin and placed in 70% ethanol, paraffin-embedded sectioned to 2 µm for immunohistochemical and morphometric analyses.

Lung histology

After acute or chronic normoxia or hypoxia animals were euthanized and the organs collected. For hypoxic animals this was done inside an air controlled chamber to prevent reoxygenation. Lungs were fixed in 10% buffered formalin, embedded in paraffin and cut in 2-3 µm sections and stained with hematoxylin and eosin (H&E). Additionally immunohistochemistry was done with the primary alpha smooth muscle actin (αSMA), a pre-diluted monoclonal mouse antibody (DAKO, Glostrup, Denmark, N1584). In brief, lung sections were incubated with this primary antibody (30 min, RT), rinsed with TBS (DAKO wash Buffer, S3006), and as secondary antibody labelled with peroxidase was used the detection kit ChemMate (3-Amino-9-Ehtylcarbazol (AEC) from DAKO (K5003)). The slides were incubated with the second antibody (10 min, RT) and sections were counterstained with hematoxylin. A second set of sections was H&E-stained to evaluate oedema.

Quantitative procedures

Lung sections were analysed using StereoInvestigator software (MBF Bioscience, Williston, Vermont). The areas of the section were defined as regions of interest. Within this area, a uniform random systematic set of sampling locations was selected at 500 × 500 µm intervals along the x- and y- axis. At each sampling location, a 150 × 150 µm unbiased counting frame (11) was used to tally the intersections of blood vessels with the plane of the section. At the same time, vessels were classified as non-, partially- or fully-muscularized (Figure 4). Within the area defined by the counting frame, the area fraction fractionator was used to estimate the volume of vascular smooth muscle and total vessel volume (without adventitia). The approach combines fractionator sampling with the Cavalieri estimator of volume (10). For the latter, we used a 10 × 10 µm point grid.

Media thickness is a commonly used parameter to describe changes in vessel structure. It is however difficult to provide replicable criteria for this measurement in view of the different extent of muscularization of the vessels and the different angles at which they penetrate the section. We therefore calculated media thickness based on estimates of media volume, non-media volume and vessel length. Vessel length (L) was calculated from the number of intersection of vessels (Q, equivalent to the number of vessels counted multiplied by the area sampling fraction) section area (A) and the volume of the section (V_{ref}): $L = 2Q/A \times V_{ref}$ (10). Media thickness was calculated as the difference in the radius of a cylinder with total volume (media and non-media) and length L and the radius of a cylinder with non-media volume and length L.

Pulmonary Vascular Protein Extravasation

As previously described, pulmonary vascular leak was assessed by lung protein extravasation determined by the Evans blue dye method (16). Briefly, Evans blue dye (20 mg/kg) was injected via the venous catheter 15 minutes prior to euthanasia with an overdose of sodium pentobarbital (100 mg/kg) via the jugular catheter. Animals were then transcardially perfused with phosphate buffered saline. The left lung lobe was then perfused-fixed for immunohistochemical analyses (see below). The right lung lobe was then removed. After weighing, the caudal lobe was frozen in liquid nitrogen for extraction of Evan's blue dye. At a later time, Evans blue dye was extracted from the caudal lobe by formamide (100%) incubation (24 h, 37°C). The medial lobe was immediately weighed, oven dried (65°C, 48 h), and weighed again. Wet weight: dry weight ratio in the medial lobe was used to estimate the dry weight of the caudal lobe. The extracted dye was quantified in a spectrophotometer by measuring the absorbance at 600 nm against standards of Evans blue dye dissolved in formamide. Evans blue dye extravasation is expressed as nanograms of Evan's blue dye per milligram of dried tissue.

Plasma Epo and VEGF

Plasma, collected from heparinized whole blood, was analysed for erythropoietin (Epo) and vascular endothelial growth factor (VEGF) using ELISA methodology as previously described (R&D Systems, Minneapolis, MN, USA, Catalogue Numbers MEP00B and RRV00 respectively) (31).

HIF-1alpha DNA-binding

Nuclear protein from HPAEC was prepared using a Clontech TransFactor Extraction Kit (Palo Alto, CA, USA) and protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA*).* HIF-1 DNA-binding was then determined using the Clontech Transfactor Colorometric Kit (Palo Alto, CA, USA), as previously described (30).

Statistics

Statistical analysis and presentation follow the guidelines of the American Physiological Society (5). A two-way ANOVA (SPSS 19, IBM SPSS Statistics or GraphPad Prism 4) was used to evaluate strain and treatment differences and interactions between strain and treatment. P-values less than 0.05 were considered significant.

5. Results

Blood gas analyses

Acute hypoxia

Blood gas values differed between strains; however the effect of hypoxia was obvious (Table 2). The partial pressure of oxygen (pO_2) ($p<0.0001$), oxygen saturation (SAT) ($p<0.0001$) and partial pressure of carbon dioxide (pCO_2) ($p<0.0001$) were all significantly lower in hypoxia compared to normoxia. Furthermore, pH was increased ($p=0.0002$) in hypoxia compared to normoxia.

Chronic hypoxia

On the other hand, after 21 days of hypoxic exposure pO_2 and pCO_2 were normalized in both strains, but oxygen saturation in both strains was lower with hypoxia ($p=0.002$) and with H being significantly lower than M ($p=0.04$) (Table 3). pH values were lower after chronic hypoxia ($p<0.0001$) and again to a larger extent in H rats ($p=0.017$).

Oxygen capacity

Acute Hypoxia

There was no effect of acute hypoxia on hemoglobin (Hb) and O_2 capacity (O_2cap), yet higher values were observed in hypoxic H compared with M rats (Hb $p=0.032$ / O_2cap $p=0.035$) (Table 3). Reduced Hb was, as expected, higher under hypoxia ($p<0.0001$) in both strains, and hematocrit was unchanged.

Chronic Hypoxia

In contrast, Hb and O_2 cap were elevated following chronic hypoxia in both strains with the greatest increase in H rats (Table 3). Similar findings were observed for reduced Hb and HCT; all were elevated with chronic hypoxia, yet hypoxic H rats had significantly greater values than M rats.

Systemic pressures

Acute Hypoxia

There were no changes in systemic pressures in response to acute hypoxia (Table 2).

Chronic Hypoxia

After 21 days of hypoxia systemic diastolic pressure, total systemic resistance (TSR) and heart rate were not significantly different, whereas systemic mean pressure mean ($p=0.029$),

systemic systolic pressure (p=0.037) and cardiac output (p=0.042) were all lower than in normoxic conditions (Table 3).

Pulmonary artery pressure

Acute Hypoxia

Once exposed to acute hypoxia, both rat strains showed elevated pulmonary artery pressure (Ppa) ($p<0.0001$), whereas the total pulmonary resistance (TPR) did not increase significantly (p=0.0847) (Figure 1).

Chronic Hypoxia

Chronic hypoxic exposure resulted in elevated PPA and TPR ($p<0.0001$), and PPA was significantly greater in hypoxic H as compared with M rats (p=0.0064 (Table 3).

Right ventricular hypertrophy

Acute Hypoxia

Although PPA rose with acute hypoxia, the time of exposure was too short to allow for cardiac hypertrophy.

Chronic Hypoxia

In lieu of the fact that PPA and TPR were significantly elevated following chronic hypoxia, the finding of right ventricular (RV) hypertrophy was expected and observed in both strains (Figure 2).

Pulmonary edema

Acute Hypoxia

Because acute hypoxia is associated with the development of pulmonary edema, associated with high pulmonary artery pressures, we assessed pulmonary leak using Evan's blue dye. Pulmonary leak was detected in M but not in H rats after acute hypoxic exposure (Figure 3).

Chronic Hypoxia

However, after 21days of hypoxia, the findings were reversed, pulmonary leak was observed in H, but not in M rats. Histological evidence supported the findings from the Evan's blue dye studies, elevated numbers of alveolar macrophages in the alveolar spaces, as compared with normoxic animals. Additional evidence of edema from histological studies included the presence of clear fluid in widened interstitium around bronchi, bronchioles, blood vessels and

interlobar septae. Furthermore, in chronic hypoxic H rats, signs of occlusion in small vessels and a few thrombi in larger vessels were detected.

Pulmonary vascular remodelling in response to chronic hypoxia

The acute hypoxia exposures were not of sufficient length to for pulmonary vascular remodelling to develop. Therefore, data were only collected from chronically hypoxic and normoxic animals.

Rigorous stereological analyses of lung sections were conducted to determine the exact nature of hypoxic effects on the pulmonary vasculature (Figure 4, and Table 4). H rats had greater volume ($p=0.001$) and thickness ($p<0.001$) of vascular media than the Madison rats under normoxic conditions, but non-medial layers had similar characteristics (Table 4). In both strains, chronic hypoxia was associated with greater volume of the media ($p<0.001$) and t medial thickness ($p<0.001$), while non-medial layers were not affected ($p=0.212$).

Vessel length in the sections was not different between the two strains ($p=0.767$) but differed for the treatments ($p=0.017$). Assessed areas of lung sections were not different between the two strains ($p=0.203$) or between the treatments (normoxia and hypoxia, $p=0.072$).

Under normoxic conditions, a greater percent of pulmonary vessels in the H rat were fully-muscularized ($p<0.001$) and a lesser percent non-muscularized ($p=0.03$), than in the M rats (Figure 5). In response to chronic hypoxia, both a greater percent of vessels were fully-muscularized and a lesser percent non-muscularized compared with normoxic values. Chronically hypoxic H rats had significantly more fully-muscularized vessels ($p=0.017$) than M animals.

Plasma Epo and VEGF

Plasma Epo and VEGF were evaluated because hypoxia is associated with elevations in plasma Epo and VEGF, and because Epo and VEGF have been implicated in elevations of pulmonary artery pressure and pulmonary vascular remodelling.

Acute Hypoxia

Acutely hypoxic H rats had significantly greater plasma Epo and VEGF than the normoxic H rats, however Epo and VEGF were not greater hypoxic as compared with normoxic M rats (Figure 6).

Chronic Hypoxia

Chronically hypoxic H and M rats had greater Epo than normoxic rats, while only H rats had greater plasma VEGF. In fact, Epo and VEGF in chronically hypoxic H rats were even greater than that in hypoxic M rats.

Pulmonary HIF-1alpha binding

Because H and M rats showed distinct differences in pulmonary and hemodynamic responses to acute and chronic hypoxia which were further reflected in varying concentrations of associated factors plasma Epo and VEGF, analyses of the primary factor responsible for hypoxia-induced increases in Epo and VEGF, binding of hypoxia-inducible transcription factor -1 (HIF-1) to its DNA site was evaluated in the lungs.

Acute Hypoxia

HIF-1 DNA-binding in the nuclei of pulmonary cells was only elevated in H rats exposed to chronic hypoxia (Figure 6).

Chronic Hypoxia

After 21d of hypoxia, M rats showed no sign of elevated HIF-1 DNA-binding, yet chronically hypoxic H rats experienced very high levels of binding.

6. Discussion

The data support previous findings of significantly divergent responses to acute and chronic hypoxia in Madison and Hilltop rats. Further investigation implicates hypoxia-inducible transcription factor -1 (HIF-1) in the variable physiologic responses between strains. Interestingly, differences in response to high altitude between diverse human populations have been attributed in part to EPAS1, the gene encoding HIF-2 (2, 29, 32).

While some responses to hypoxia between the two strains are similar, such as equivalent decreases in PO_2, PCO_2, pH, or oxygen content associated with increases in pulmonary artery pressure (Ppa) in response to acute hypoxia**,** others are quite different, such as acute hypoxia only causing pulmonary edema in Madison rats and chronic hypoxia only causing pulmonary edema in Hilltop rats.

Acute hypoxia

In response to acute hypoxia, H and M rats had similar and expected decreases in PO_2, PCO_2, pH, Hb and oxygen content. They also had similar increases in hemoglobin and calculated oxygen capacity. Ppa was elevated in both strains, while TPR, cardiac output (CO), heart rate (HR) and systemic blood pressure remained unaltered. Despite all of these equivalent physiologic changes, only M rats developed pulmonary leak, H rats did not. On a molecular level, the apparent protection from acute hypoxia-induced pulmonary leak in H rats was associated with elevated plasma Epo, VEGF and pulmonary HIF-1 DNA-binding activity. In recent years, Epo has been implicated in many non-erythroid functions (8) including cellular protection from apoptosis (1, 23). Pulmonary leak involves apoptosis (22, 28), thus Epo may be contributing to the protection of the lungs from leak during hypoxia in H rats. Further, VEGF has been reported as possibly protective in high altitude pulmonary edema (12, 21). The higher HIF-1 activity in H as compared with M acutely hypoxic rats likely contributes to the elevated plasma Epo and VEGF.

Chronic Hypoxia

The chronic hypoxia blood chemistry data are collected under anesthesia due to the complications of maintaining patent catheters for 21 days. However, there were significant differences in blood chemistry between H and M rats exposed to chronic hypoxia. While PO_2, PCO_2 and oxygen content remained equivalent to that of normoxic animals, pH, O_2 saturation, Hb were significantly lower in rats exposed to chronic hypoxia and HCT, Hb and oxygen

capacity increased compared to normoxic animals. Interestingly, hypoxic H rats had significantly lower pH and greater HCT, Hb than M rats, likely contributing to the large calculated oxygen capacity in chronically hypoxic H rats.

Both strains demonstrated pulmonary hypertension following chronic hypoxia, yet H rats had much more dramatic mean pulmonary artery pressures, primarily due to a large increase in the systolic pressure, while total pulmonary resistance remained elevated to the same extent in both hypoxic strains. Of note, CO and HR remained unchanged. The pulmonary hypertension likely led to the development of right ventricular hypertrophy and accordingly the H strain, which had the greatest pulmonary artery pressures, had significantly more right ventricular hypertension.

Unlike high altitude pulmonary edema, which occurs in humans shortly after arrival at high altitude, H rats only developed pulmonary leak after 21d at high altitude, not following acute hypoxia. These data do not address the mechanism of chronic hypoxia-induced pulmonary leak in H rats. However, the molecular data suggest a potential mechanism. Plasma Epo and VEGF, and pulmonary HIF-1 activity remained elevated following 21d hypoxia in H rats, however in M rats only plasma Epo remained elevated, yet in a significantly lower concentration than in H rats. The prolonged elevation of plasma Epo and VEGF, and pulmonary HIF-1 activity may contribute to the development of pulmonary leak. HIF-1 activation and the resulting increase in expression of proteins such as Epo and VEGF are believed to protect the system from hypoxia, Epo by elevating erythropoiesis, VEGF by promoting angiogenesis (3), however prolonged elevation of HIF activity may have negative consequences such as pulmonary hypertension and remodelling (27).

Pulmonary vascular remodelling

As has been previously demonstrated (17, 20), H rats develop robust pulmonary hypertension and right ventricular hypertrophy in response to chronic hypoxia, which is associated with pulmonary vascular remodelling. While the pulmonary vasculature of M rats had some evidence of remodelling in response to chronic hypoxia, it was much more pronounced in H rats. While previous studies have identified the extensive pulmonary remodelling in H rats exposed to chronic hypoxia, using the advantage of cutting edge stereologic methods, a more detailed investigation of the changes in pulmonary vessels was undertaken in the current study than was possible when the original study was conducted (17).

The initial finding was that the vascular medial layer, but not non-medial layers differed between the two strains and between normoxic and hypoxic animals. Normoxic H animals had a thicker medial layer than normoxic M animals, yet exposure to chronic hypoxia resulted in H and M rats have similar increases in media thickness and volume. Interestingly the percentage of all vessels that were fully-muscularized was greater in H as compared with M rats in normoxic control conditions, while the percent of non-muscularized vessels was less. Exposure to chronic hypoxia increased the percent of fully muscularized vessels in both strains, yet the largest increase was in H rats.

HIF-1, Epo and VEGF have all been implicated in hypoxia-induced pulmonary vascular hypertension remodelling (6, 13, 24) and may well be playing a role in the extensive remodelling noted in the H as compared with M rats following chronic hypoxic exposure.

Caveats

Comparisons between acute and chronic data are difficult due the fact that animals in the acute study had patent catheters and data were collected awake animals, however it was not possible to keep catheters patent over 21days, thus animals were anesthetized during data collection. It was not possible to administer hypoxia during anesthesia; therefore all chronic data are collected during normoxia. Stereologic data should be interpreted as the result of chronic hypoxia, while blood gas values and HIF activity data must be interpreted as responses to normoxic anesthesia following exposure to chronic hypoxia.

Conclusion

HIF-1 activity is associated with physiologic responses to acute hypoxia and pathophysiologic responses to chronic hypoxia in Hilltop rats. The highly variable physiologic responses to acute and chronic hypoxia between Madison and Hilltop rats are complex, yet may prove valuable to further understanding the divergent responses of individuals and ethnic groups to acute and chronic hypoxia. Indeed, very recent studies have revealed genetic determinants in Tibetans living at high altitude exist (reviewed in (29)), but the mechanisms that allow adaptation to high altitude remains unknown.

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8. Figure legends

Table 1. Male Madison Sprague-Dawley and Hilltop rats were randomly assigned to 24h or 21d normoxic or hypoxic conditions (n=40 total animals, n=5 per group). Animals in the 21d experiments were catheterized at the completion of the exposure time and hemodynamic measurements were obtained under anesthesia (see materials and methods).

Table 2. Blood gases, heart rate, systemic and pulmonary blood pressures, and cardiac output were determined for 10 min under experimental conditions in Madison and Hilltop rats (acute (24h) normoxia or hypoxia) *significantly different from normoxic value within same strain ($p < 0.05$), #significantly different from hypoxic value in different strain ($p < 0.05$).

Table 3. Blood gases, heart rate, systemic and pulmonary blood pressures, and cardiac output were determined for 10 min under anesthesia in Madison and Hilltop following exposure to chronic (21d) normoxia or hypoxia. *significantly different from normoxic value within same strain ($p < 0.05$), #significantly different from hypoxic value in different strain ($p < 0.05$).

Table 4: Media thickness for pulmonary vessels was calculated based on estimates of media vessel volume, non-media vessel volume and vessel length in Madison and Hilltop rats following exposure to chronic hypoxia. *significantly different from normoxic value within same strain ($p < 0.05$), #significantly different from hypoxic value in different strain ($p < 0.05$).

Figure 1: Pulmonary hemodynamics were measured after exposure to both, acute and chronic hypoxia. *a)* Systolic (white bars), diastolic (black bars) and mean (grey bars) pulmonary pressure after acute hypoxia, *b)* pulmonary pressures after chronic hypoxia and total pulmonary vascular resistance after *c)* acute, and *d)* chronic hypoxia. *significantly different from normoxic value within same strain ($p < 0.05$), #significantly different from hypoxic value in different strain ($p < 0.05$).

Figure 2: Right ventricular hypertrophy was determined by the ratio of right ventricle (RV) and left ventricle plus septum (LV+S) in Madison and Hilltop rats exposed to chronic hypoxia. *significantly different from normoxic value within same strain ($p < 0.05$), #significantly different from hypoxic value in different strain ($p < 0.05$).

Figure 3: a) Evan's Blue dye was employed to determine the presence of pulmonary edema following exposure to acute or chronic hypoxia (and normoxic controls) in Madison and Hilltop rats. *b)* Lung sections stained with hematoxylin and eosin (H&E) were analysed for the presence of edemic fluid. *significantly different from normoxic value within same strain ($p < 0.05$), #significantly different from hypoxic value in different strain ($p < 0.0001$).

Figure 4: *a)* Following chronic hypoxia, pulmonary vascular remodelling was assessed using immunohistochemical methods to identify smooth muscle cells in Madison and Hilltop rats. *b)* Quantitative analyses were performed using StereoInvestigateor software (MBF Bioscience, Williston, Vermont). *significantly different than all other values ($p < 0.05$).
Figure 5: Immunohistochemistry was used to identify pulmonary vascular smooth muscle cells following chronic hypoxia in Madison and Hilltop rats. *a)* non-muscularized *b)* partially-muscularized and *c)* fully-muscularized vessels. *d)* Quantitative analyses were performed using StereoInvestigateor software (MBF Bioscience, Williston, Vermont). *significantly different from normoxic value within same strain ($p < 0.05$), #significantly different from hypoxic value in different strain ($p < 0.05$).
Figure 6: a) Plasma concentrations of erythropoietin (Epo), *b)* vascular endothelial growth factor (VEGF) in M and H rats were determined following exposure to acute or chronic hypoxia (normoxic controls) and *c)* nuclear proteins extracted from whole lung tissue were analysed for HIF-1 DNA-binding using the Clontech Transfactor Colorimetric kit in H and M rats exposed to acute or chronic hypoxia (normoxic controls). *significantly different from normoxic value within same strain ($p < 0.05$), #significantly different from corresponding value in different strain ($p < 0.05$), +significantly different from acute hypoxic value within same strain ($p < 0.05$).

9. Tables and figures

Table 1

Strains	n	Time of Exposure	% O_2 during hemodynamic measurements	Simulated altitude during experiment	Anesthesia during hemodynamic measurements
Madison	5	24 h	18%	1500 m	No
Madison	5	24 h	10%	5500 m	No
Hilltop	5	24 h	18%	1500 m	No
Hilltop	5	24 h	10%	5500 m	No
Madison	5	21 d	21%	1500 m	Yes
Madison	5	21 d	21%	5500 m	Yes
Hilltop	5	21 d	21%	1500 m	Yes
Hilltop	5	21 d	21%	5500 m	Yes

Table 2

	Madison		*Hilltop*	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Body Weight (g)	325 (16)	327 (21)	323 (18)	343 (30)
pH	7.48 (0.018)	7.55 (0.014)*	7.47 (0.035)	7.55 (0.01)*
PCO_2 (mm Hg)	33.3 (0.96)	18.5 (0.71)*	30.4 (5.18)	15.5 (2.38)*
PO_2 (mm Hg)	75 (1.9)	47 (0.7)*	83 (8.5)	42 (10.5)*
Hb (g/dl)	12.9 (0.41)	12.3 (0.21)	14.0 (0.54)	14.7 (1.66)#
O_2 saturation (%)	91 (5)	59 (2)*	91 (3)	57 (8)*
O_2 content (vol %)	16.3 (1.26)	8.2 (3.56)*	17.6 (1.07)	11.2 (1.16)*
O_2 capacity (vol %)	17.9 (0.56)	17.0 (0.03)	19.4 (0.75)	20.4 (2.41)#
Heart Rate (bpm)	398 (28)	377 (66)	428 (22)	358 (72)
CO (L/min)	0.387 (0.097)	0.394 (0.216)	0.361 (0.087)	0.403 (0.166)
Systemic Mean (mm Hg)	129 (8)	123 (12)	124 (12)	112 (9)
Systemic Systolic (mm Hg)	147 (11)	143 (17)	144 (13)	128 (19)
Systemic Diastolic (mm Hg)	112 (10)	106 (14)	106 (11)	96 (7)
TSR (mm Hg)	2354 (861)	2420 (948)	2333 (526)	2135 (879)

Table 3

	Madison		*Hilltop*	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Body Weight (g)	382 (10)	349 (17)	403 (21)	330 (32)*
HCT (%)	49 (1)	64 (4)*	49 (2)	71 (5)*#
pH	7.39 (0.019)	7.31 (0.042)*	7.37 (0.015)	7.25 (0.033)*#
PCO_2 (mm Hg)	43 (2)	45 (8)	43 (3)	46 (4)
PO_2 (mm Hg)	67 (9)	64 (14)	62 (2)	58 (8)
Hb (g/dL)	12.4 (1.18)	16.9 (2.38)*	12.9 (1.08)	20.8 (2.42)*#
O_2 Saturation (%)	84 (5)	64 (6)*	78 (4)	56 (5)*
O_2 Content (vol %)	14.5 (2.04)	15.3 (1.27)	13.9 (1.17)	16.3 (2.76)
O_2 Capacity (vol %)	17.2 (1.64)	24.1 (4.38)*	17.9 (1.51)	29.3 (3.62)*
Heart rate (bpm)	272 (13)	263 (14)	283 (19)	270 (15)
Systemic Mean (mm Hg)	114 (10)	105 (9)	116 (9)	98 (5)
Systemic Systolic (mm Hg)	128 (10)	117 (9)	132 (17)	107 (6)*
Systemic Diastolic (mm Hg)	99 (9)	94 (9)	101 (6)	86 (5)
TSR (mm Hg)	2402 (180)	3040 (584)	2965 (532)	2291 (384)
Cardiac output (L/min)	0.310 (0.029)	0.218 (0.052)	0.289 (0.074)	0.234 (0.037)

Table 4

	Madison		*Hilltop*	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Total area (mm^2)	54.2 (6.9)	60.8 (8.5)	58.3 (13.6)	67.6 (5.7)
Vessel length (µm)	2222 (293)	3067 (337)	2347 (784)	2796 (604)
Vol media ($*10^4$ $µm^3$)	8,9 (2,8)	17,2 (2,5)	15,2 (4,2)	25,5 (5,4)
Vol non-media ($*10^4$ $µm^3$)	43,3 (10,6)	56,0 (19,4)	45,8 (16,9)	52,8 (19,3)

Figure 1

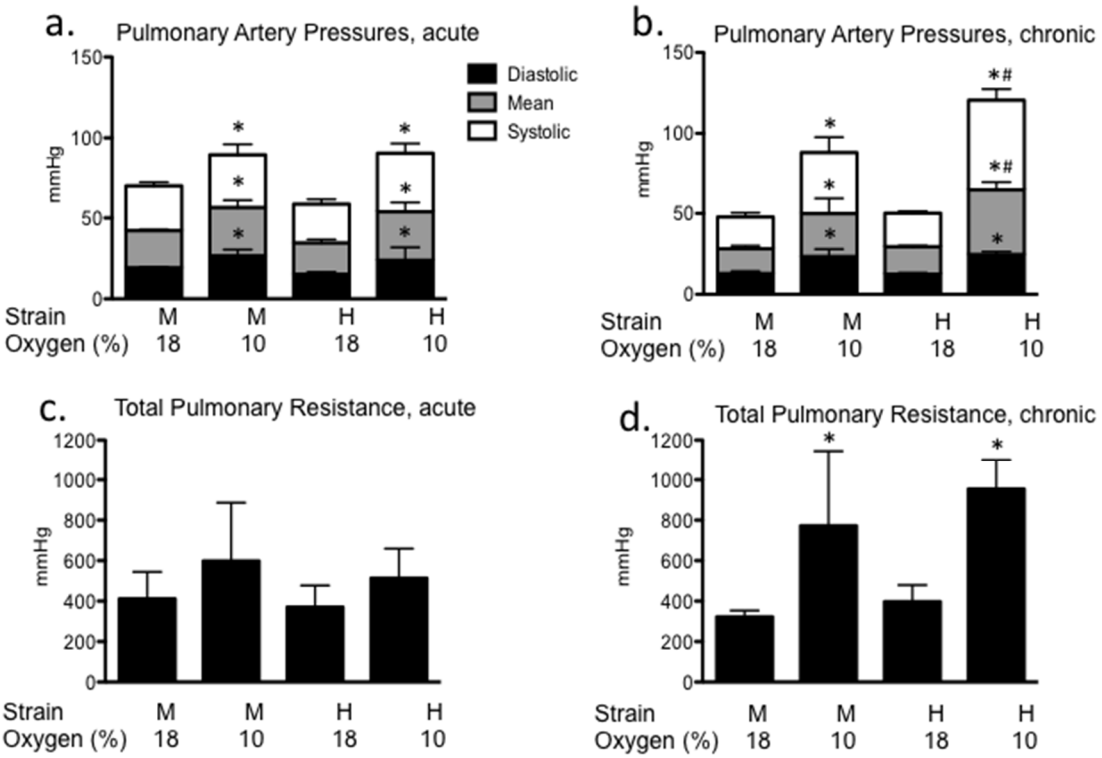

a.
Pulmonary Artery Pressures, acute
Diastolic
Mean
Systolic
mmHg
150
100
50
0
Strain
Oxygen (%)
M 18
M 10
H 18
H 10
b.
Pulmonary Artery Pressures, chronic
*#
c.
Total Pulmonary Resistance, acute
1200
1000
800
600
400
200
d.
Total Pulmonary Resistance, chronic

Figure 2

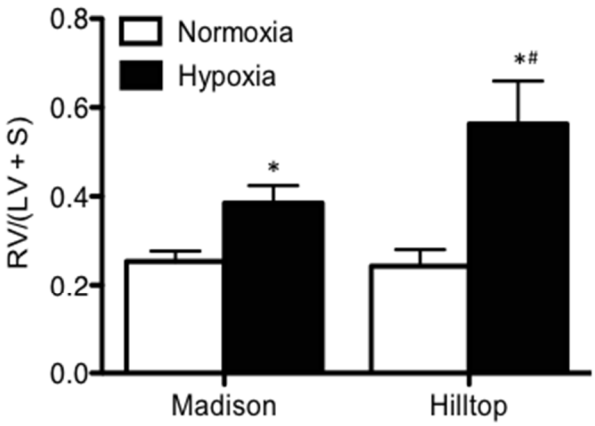


Figure 3

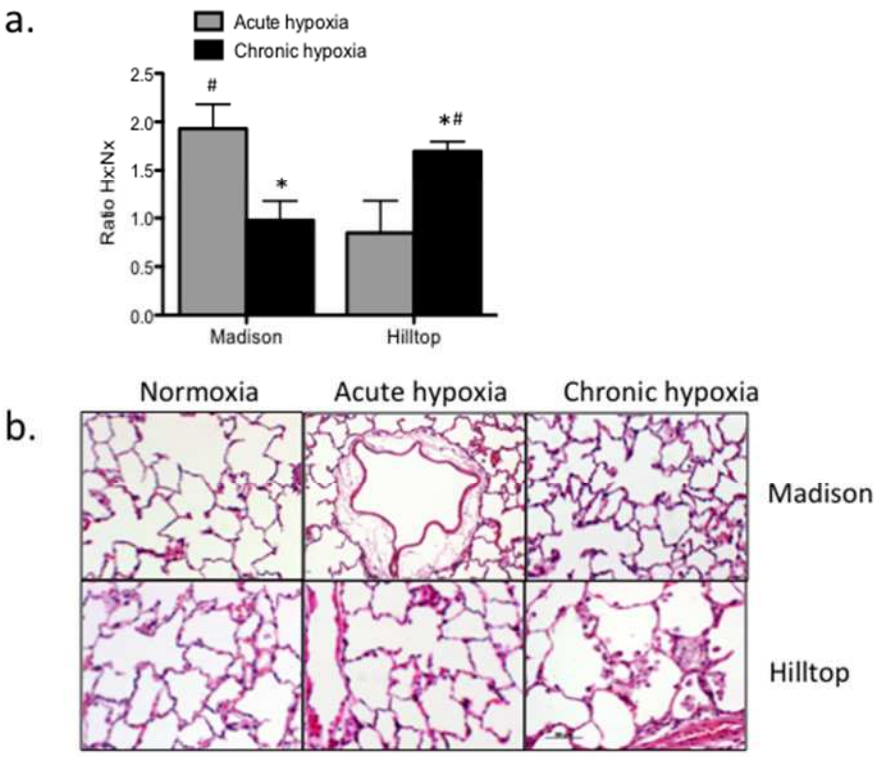

a.
Acute hypoxia
Chronic hypoxia
Ratio HxNx
2.5
2.0
1.5
1.0
0.5
0.0
#
*
*#
Madison
Hilltop
b.
Normoxia
Acute hypoxia
Chronic hypoxia
Madison
Hilltop

Figure 4

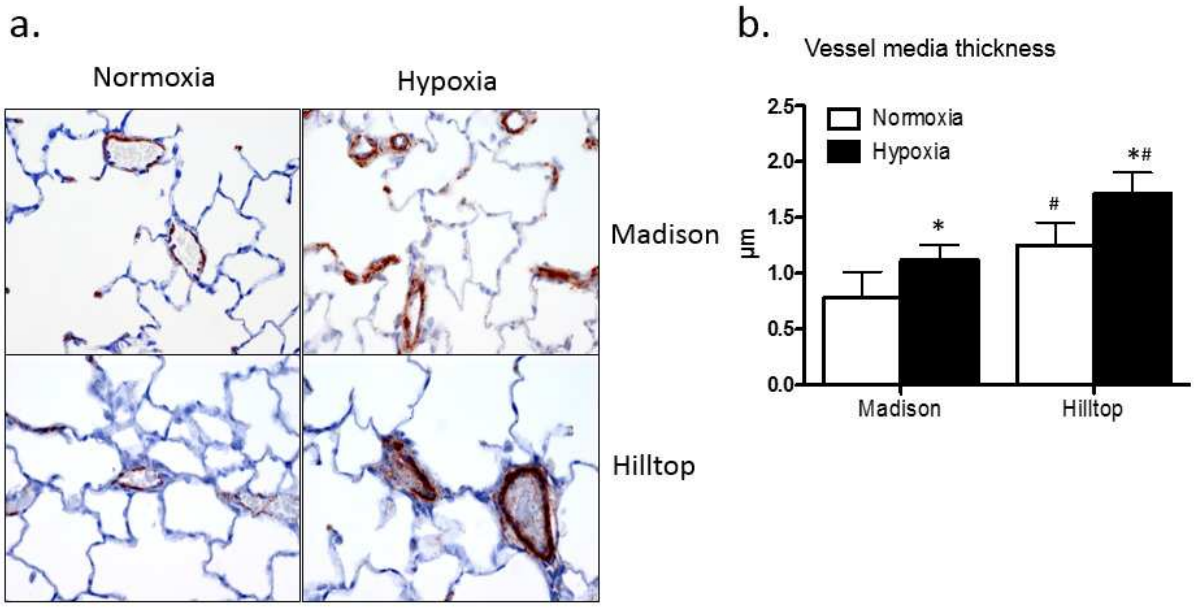

a.
Normoxia
Hypoxia
Madison
Hilltop
b.
Vessel media thickness
Normoxia
Hypoxia
μm
2.5
2.0
1.5
1.0
0.5
0.0
*
#
*#
Madison
Hilltop

Figure 5

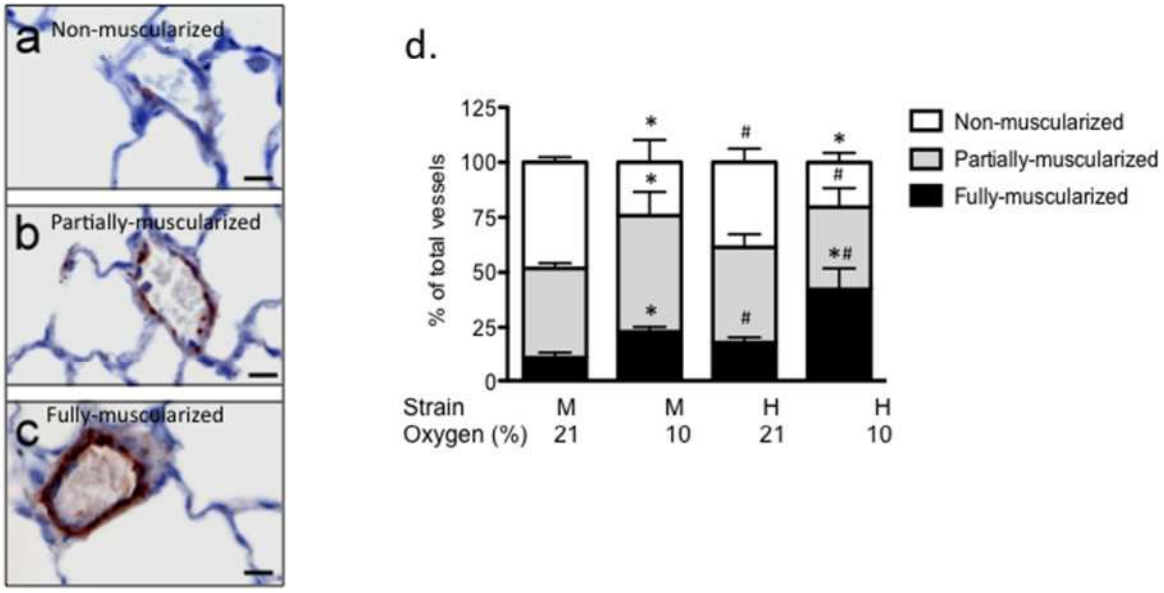
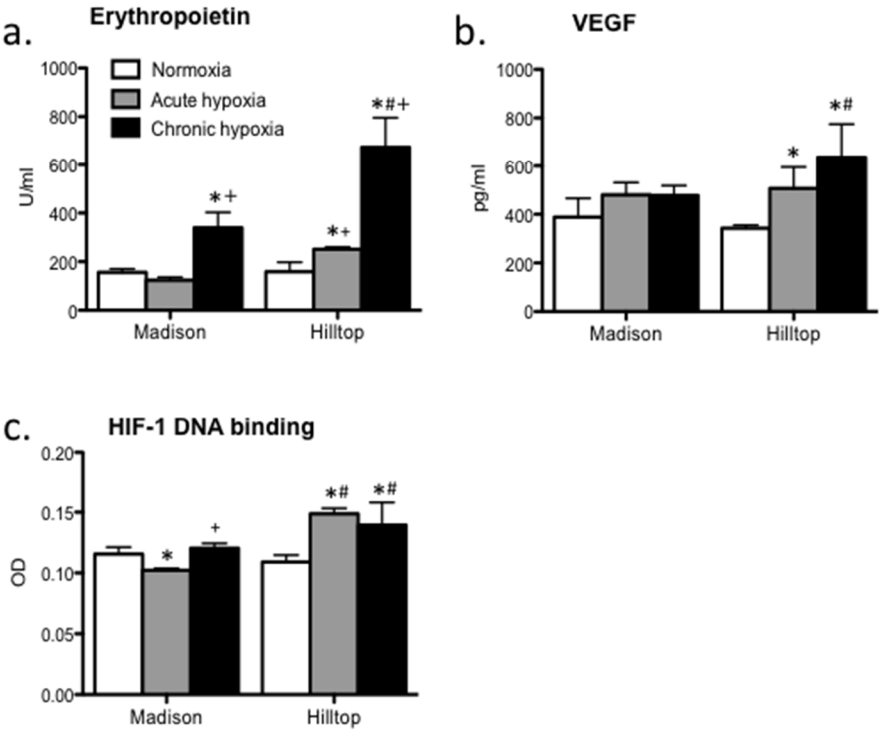

a
Non-muscularized
b
Partially-muscularized
c
Fully-muscularized
d.
% of total vessels
125
100
75
50
25
0
Strain
Oxygen (%)
M
21
M
10
H
21
H
10
Non-muscularized
Partially-muscularized
Fully-muscularized

Figure 6


a.
Erythropoietin
Normoxia
Acute hypoxia
Chronic hypoxia
U/ml
1000
800
600
400
200
0
*+
*+
*#+
Madison
Hilltop
b.
VEGF
pg/ml
1000
800
600
400
200
0
*
*#
Madison
Hilltop
c.
HIF-1 DNA binding
OD
0.20
0.15
0.10
0.05
0.00
*
+
*#
*#
Madison
Hilltop